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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John White January 31, 2003
Serial No.: 09/786,839 Group Art Unit: 1614
Filed: March 1, 2001 Examiner: Delacroix-Muirhei, C.
For: CXCR2 INHIBITORS AND PMN ADHESION AND T-CELL CHEMOTAXIS

Assistant Commissioner for Patents
Washington, D.C. 20231

THIRD DECLARATION UNDER 37 C.F.R. § 1.131(A)

I, John R. White, do hereby declare:

1. That I am the inventor of the subject matter claimed in the above referenced application;
2. That prior to December 31, 1997, the publication date of the Widdowson reference (International Publication No. WO 97/49400) and before the presumed April 1998 publication date of the White et al. reference (J. Biol. Chem. 273(17): 10095-10098), the invention claimed in the referenced application was conceived and reduced to practice in this country or a NAFTA country or a WTO member country as evidenced by the following:
 - a) Prior to December 31, 1997, SmithKline Beecham (predecessor company to GlaxoSmithKline) entered into a service agreement with Montana ImmunoTech Inc. to perform experiments designed by me to investigate the role of the IL-8B (a.k.a. CXCR2) receptor and its antagonism in neutrophil-endothelial cell adhesive interactions;
 - b) Prior to December 31, 1997, Dr. Robert F. Bargatze, Vice President & Senior Scientist at Montana Immunotech Inc. delivered to me a report on the above described investigation (a true copy of which is attached hereto as Exhibit A). The reported results

confirmed my hypothesis that compounds that bind to the CXCR2 receptor inhibit or block the binding of human neutrophils to activated endothelial cells; and

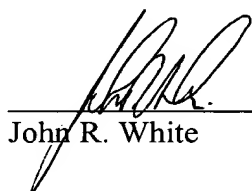
c) Prior to December 31, 1997, Judy Lee, under my direction, performed the experiments set forth at pages 29904-96 to 29904-97 of her laboratory notebook (a true copy of which is attached hereto as Exhibit B) wherein it was shown that compounds that bind to the CXCR2 receptor inhibit IL-8- or Gro α -mediated human T-cell chemotaxis in a dose dependent manner;

3. That each of the dates redacted from Exhibits A and B is before December 31, 1997;

4. That each of the acts relied upon to establish a date of conception and reduction to practice of the claimed invention prior to December 31, 1997 was performed in this country or in a NAFTA country or in a WTO member country; and

5. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

signed:


John R. White

dated:

29-Jan 03



Exhibit A

Report:
**Investigation of the role of the IL-8B
receptor and its antagonism in
neutrophil-endothelial cell adhesive
interactions.**

For:

Dr. John R. White
SmithKline Beecham plc
Dept Molecular Immunology
P.O. Box 1539
King of Prussia, PA 19406

October 21, 1997

Prepared by: Robert F. Bargatze Ph.D.
Vice President & Senior Scientist
Montana ImmunoTech Inc.
Bozeman, MT 59715
Phone: (406) 585-2733
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Project goals

The objective of this project has been to investigate, in vitro, the role of the IL-8BR in recruitment of neutrophils to 4hr IL-1 β activated HUVEC. A low nM range inhibitor specifically acting on IL-8BR will be employed to prevent IL-8BR function. This will provide a means of assessing the degree to which the IL-8BR participates in signaling dependent arrest of neutrophils on endothelial cells during flow induced shear.

The following tasks were completed

Analysis of neutrophil / 4hr activated HUVEC rolling and sticking under flow induced shear for examining the function of the neutrophil IL-8BR and the effects of the antagonist on the regulation of integrin/selectin dependent neutrophil adhesion.

Methods

Neutrophil isolation

Neutrophils were isolated from human peripheral blood. Briefly, blood was collected into citrate anti-coagulant tubes (Becton Dickinson), diluted 1:2 at room temperature in sterile HEPES (20mM) buffered HBSS (pH 7.0) (Fisher Scientific), underlaid with Histopaque 1077 and Histopaque 1117 (Sigma), and centrifuged at 2,300 RPM for 30 min at room temperature. neutrophils were collected from the Histopaque 1117/1077 interface.

Analysis of the effects the IL-8 or Gro α on the regulation of selectin/integrin mediated neutrophil rolling and arrest on IL-8 preincubated, IL-1 β activated HUVEC during flow induced shear.

The analysis of IL-8 and controls were performed in triplicate utilizing HBSS buffer containing 1% human serum buffered with 20mM HEPES. Human neutrophils were isolated, washed and infused into the shear system loop for recirculation through the capillary tube lined with confluent cultures of HUVEC pretreated with IL-8 (100ng) for 3 hours (Expts 1 and 3) or 7 hours (Expt 2), activated for 1hr with IL-1 β (10ng) followed by a 2-3 hr incubation. Gro α (5nM, 3 hr preincubation) experiments were performed using the same method as for the IL-8 preincubation experiments.

Analysis of the effects of neutrophil pretreatment with the SmithKline IL-8BR antagonist on selectin/integrin mediated neutrophil rolling and arrest on IL-8 pretreated, IL-1 β activated HUVEC during flow induced shear.

The analysis of IL-8BR antagonist were performed in triplicate in HBSS buffer

sticking. For preliminary experiments in the study neutrophils were introduced into the assay loop without HUVEC IL-8 pretreatment. In these experiments IL-8 (100ng/ml) was infused into the assay loop (or not infused) after neutrophil rolling was established on the HUVEC monolayer. Soluble IL-8 treatment during the course of these assays induced no increased sticking of neutrophils beyond that seen in the untreated controls (data not shown).

GRO_α induction of enhanced neutrophil sticking

GRO_α preincubation with HUVECS produced an effect similar to that of IL-8 for inducing neutrophil adhesion (figures 3,6). Accumulation was far above that seen for the non-GRO_α/IL-8 treated assays and shows that the CXCR2 receptor is involved in induction of enhanced neutrophil sticking during shear dependent recruitment.

SB 237844 inhibition of IL-8 enhanced neutrophil sticking

Data in three replicate experiments (figures 2, 5, 8) show that the SmithKline IL-8RB antagonist after a 10 minute preincubation with neutrophils effectively inhibits IL-8 enhanced adhesion to IL-1 activated HUVECs. One additional experiment has been performed where the same observation has been repeated but the data has not been quantified and graphed.

Flow cytometric analysis

Neutrophils showed IL-8 induced shedding of L-selectin indicating that the IL-8 was binding and triggering neutrophil activation. Interestingly, this treatment did not induce significant changes in the expression of LFA-1, MAC-1 expression as might be expected for increasing the potential for stable static neutrophil adhesion.

**Surface expression of L-selectin and LFA-1/MAC-1 on 30 minute
IL-8 incubated neutrophils as revealed by mean fluorescent levels**

| | <u>Leu-8</u> | <u>Leu-15</u> |
|-------------|--------------|---------------|
| Control | 246 | 882 |
| IL-8, 100ng | 116 | 892 |
| IL-8, 1.0ng | 100 | 816 |
| IL-8, 0.1ng | 127 | 834 |

Conclusions

- The data collected thus far in the study suggest that the SmithKline IL-8RB antagonist SB237844 is a potent inhibitor of IL-8 induced neutrophil-endothelial sticking under physiological shear conditions.
- Surprisingly, IL-8 is not an effect inducer of rapid neutrophil adhesion when administered in a soluble form after neutrophil rolling was established in the shear assay. Flow cytometry analysis did show that the IL-8 in solution did actively induced L-selectin shedding, as expected, but did not induce the expected increased expression of MAC-1/LFA-1. This observation suggested that the soluble IL-8 may not be inducing sufficient neutrophil β_2 integrin supported adhesion to cause rolling neutrophils to slow and stick on the HUVEC surface.
- Importantly, the HUVEC-IL-8 preincubation data does show that IL-8 when preincubated with HUVECs, which are subsequently activated with IL-1 β , is a potent inducer of rapid neutrophil-endothelial cell sticking that can be efficiently blocked by the SB237844 IL-8RB antagonist.

Reference:

- 1) Bargatze, R.F., S. Kurk, G. Watts, T.K. Kishimoto, C.A. Speer, and M. A. Jutila. 1994. In vivo and in vitro functional examination of a conserved epitope of L- and E-selectin crucial for leukocyte-endothelial cell interactions. J. Immunol. 152:5814.

Figure 1

Control, IL-8 preincubated HUVEC

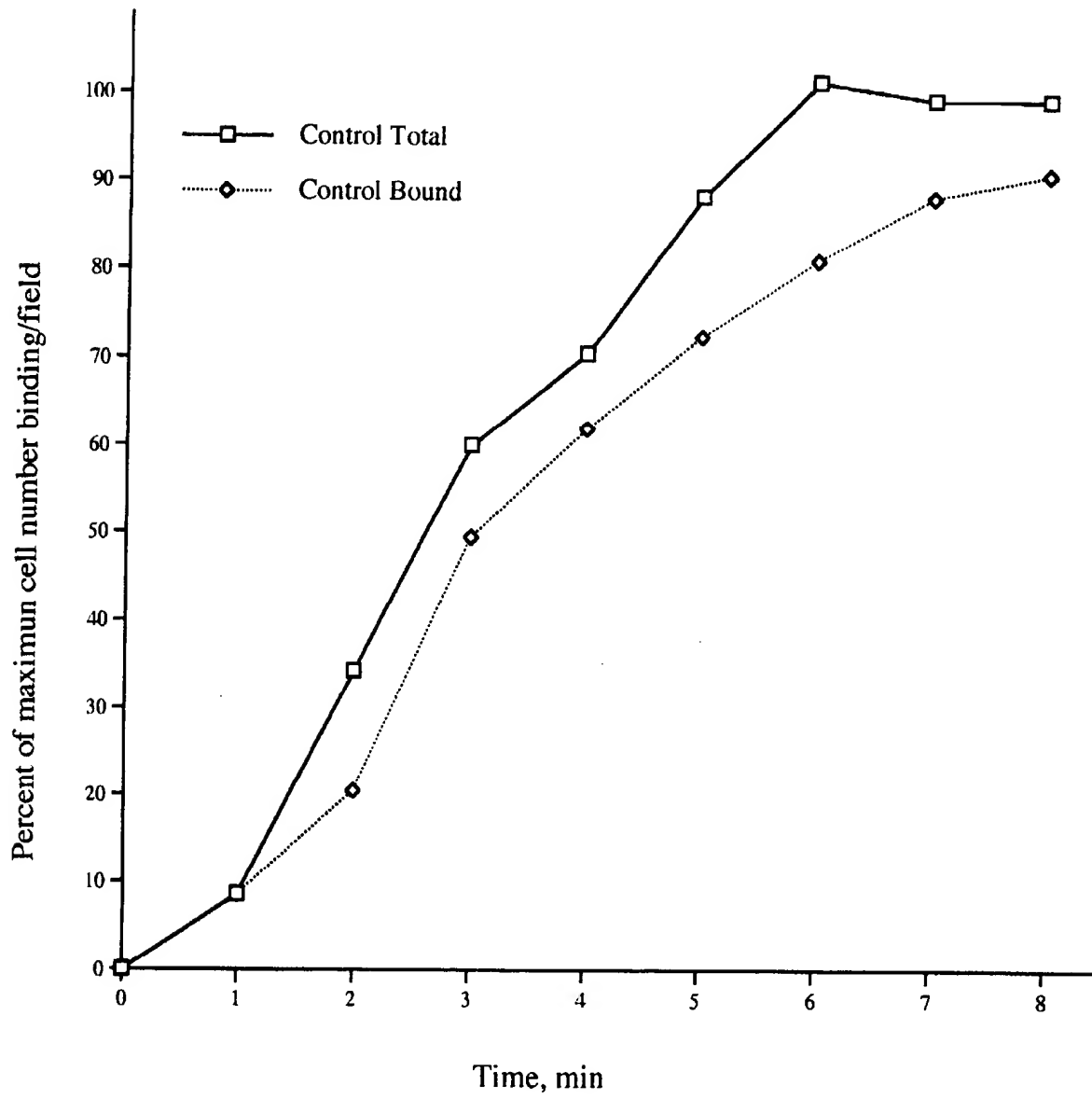


Figure 2

**SB 237844 treated neutrophils on IL-8
pretreated HUVEC**

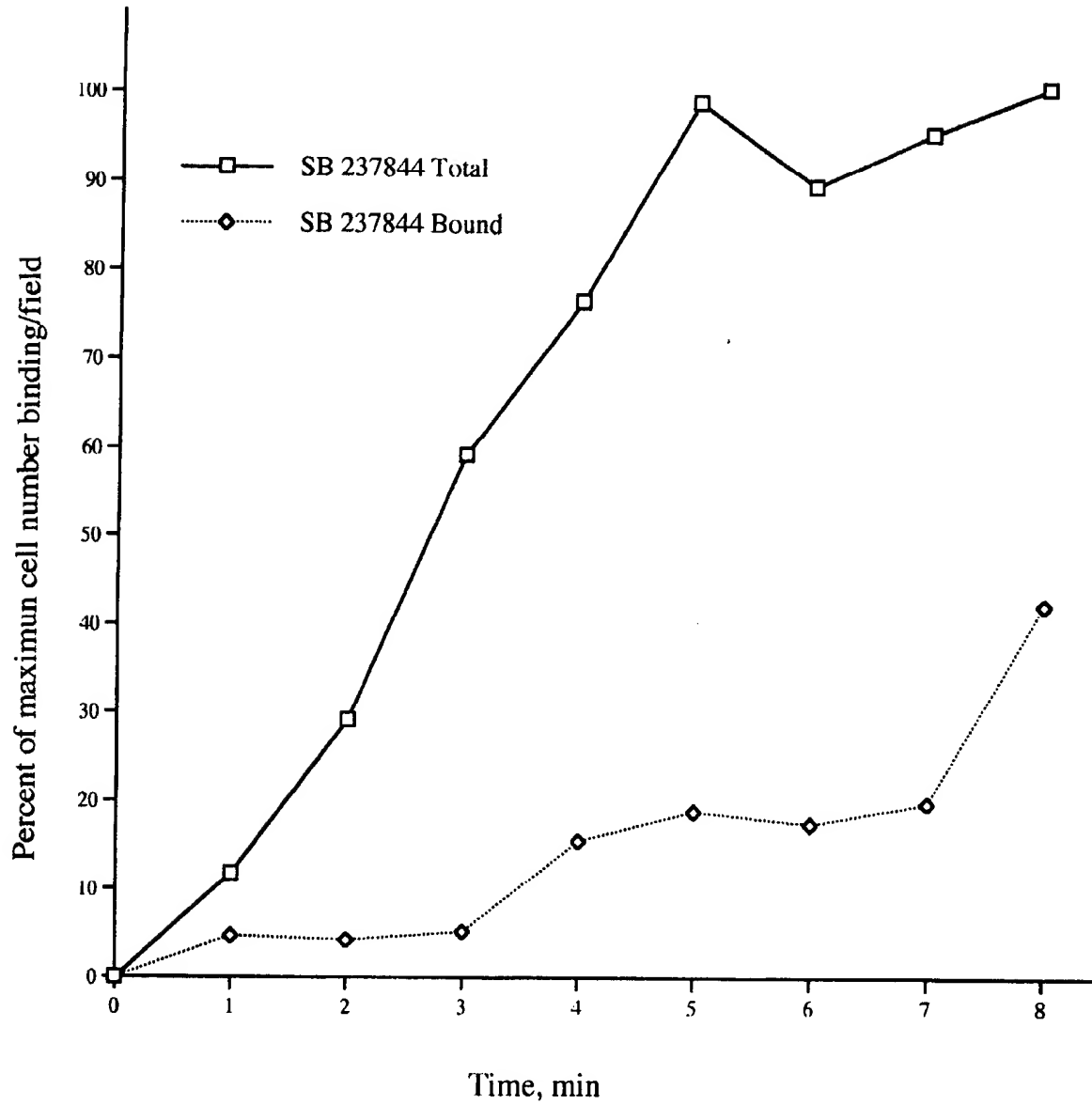


Figure 3

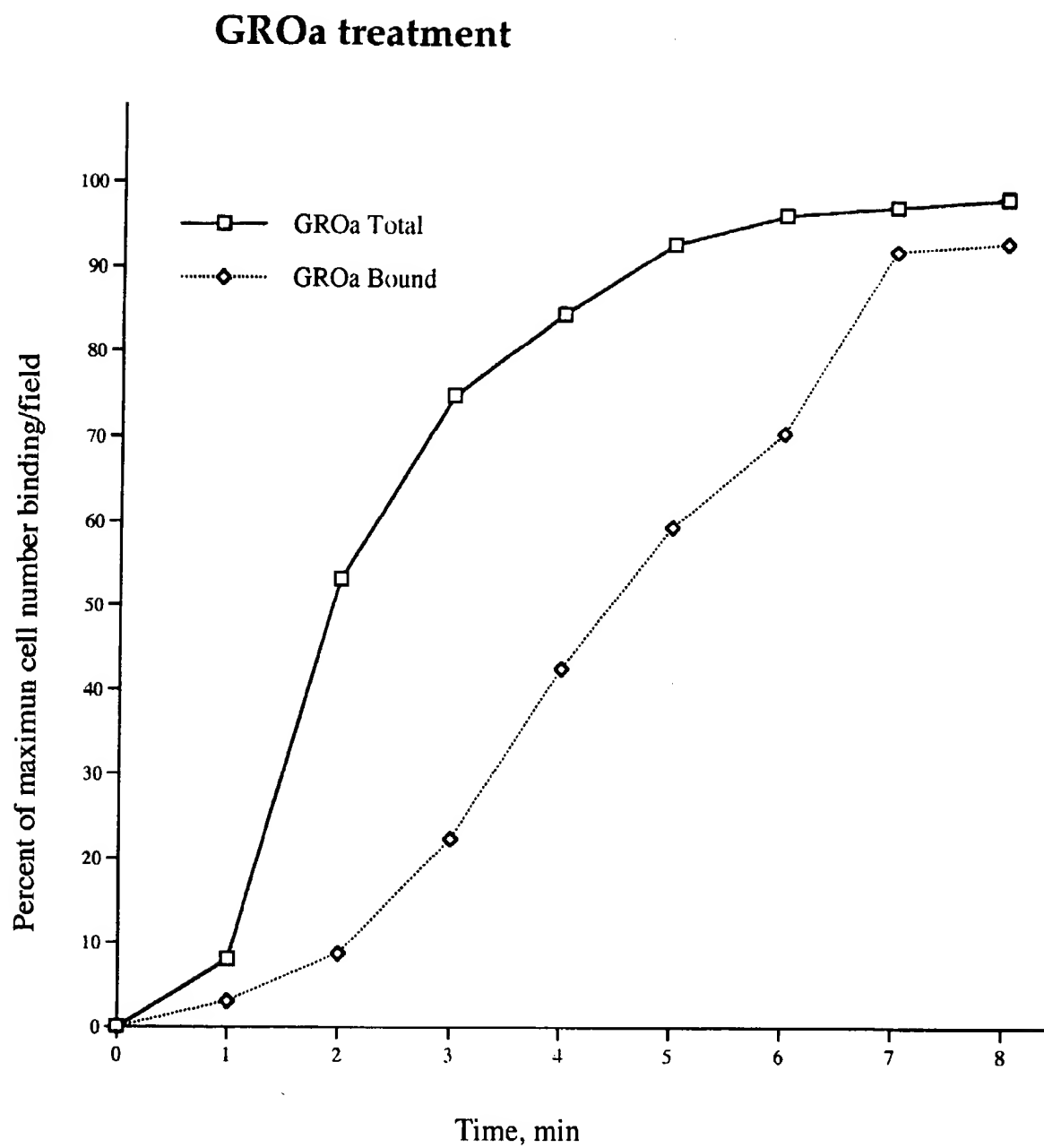


Figure 4

Control, IL-8 preincubated HUVEC

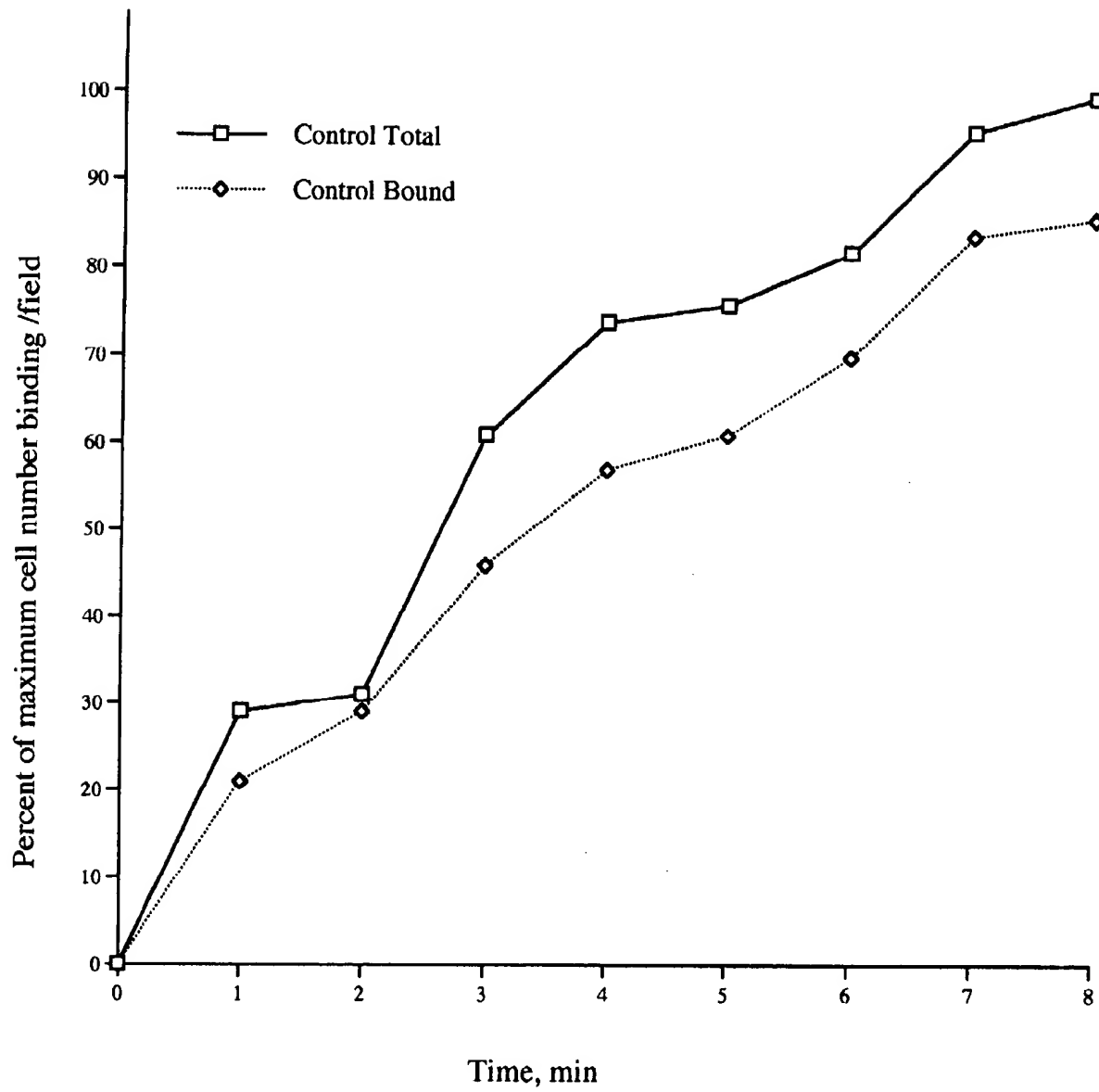


Figure 5

**SB 237844 treated neutrophils on IL-8
pretreated HUVEC**

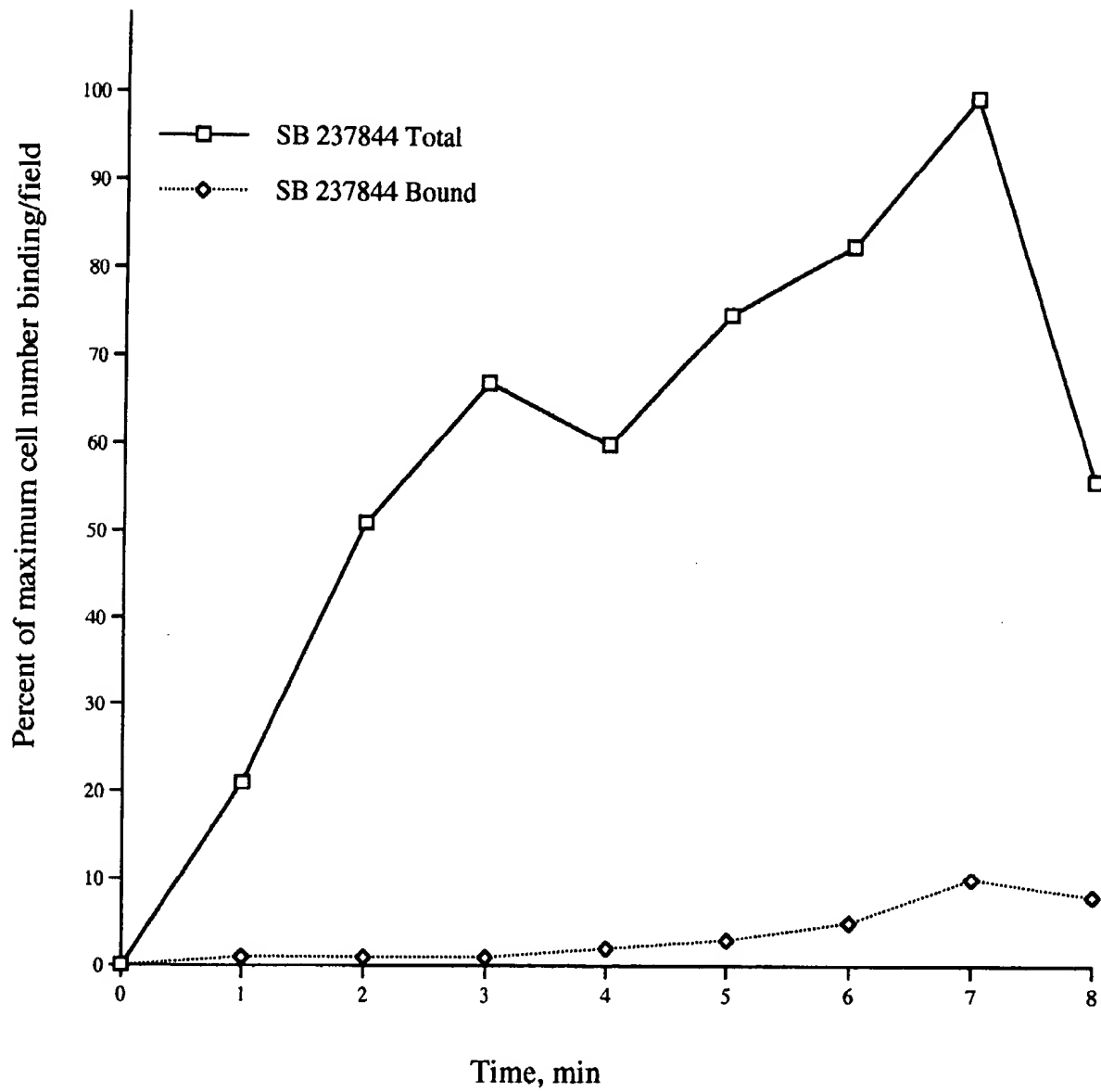


Figure 6

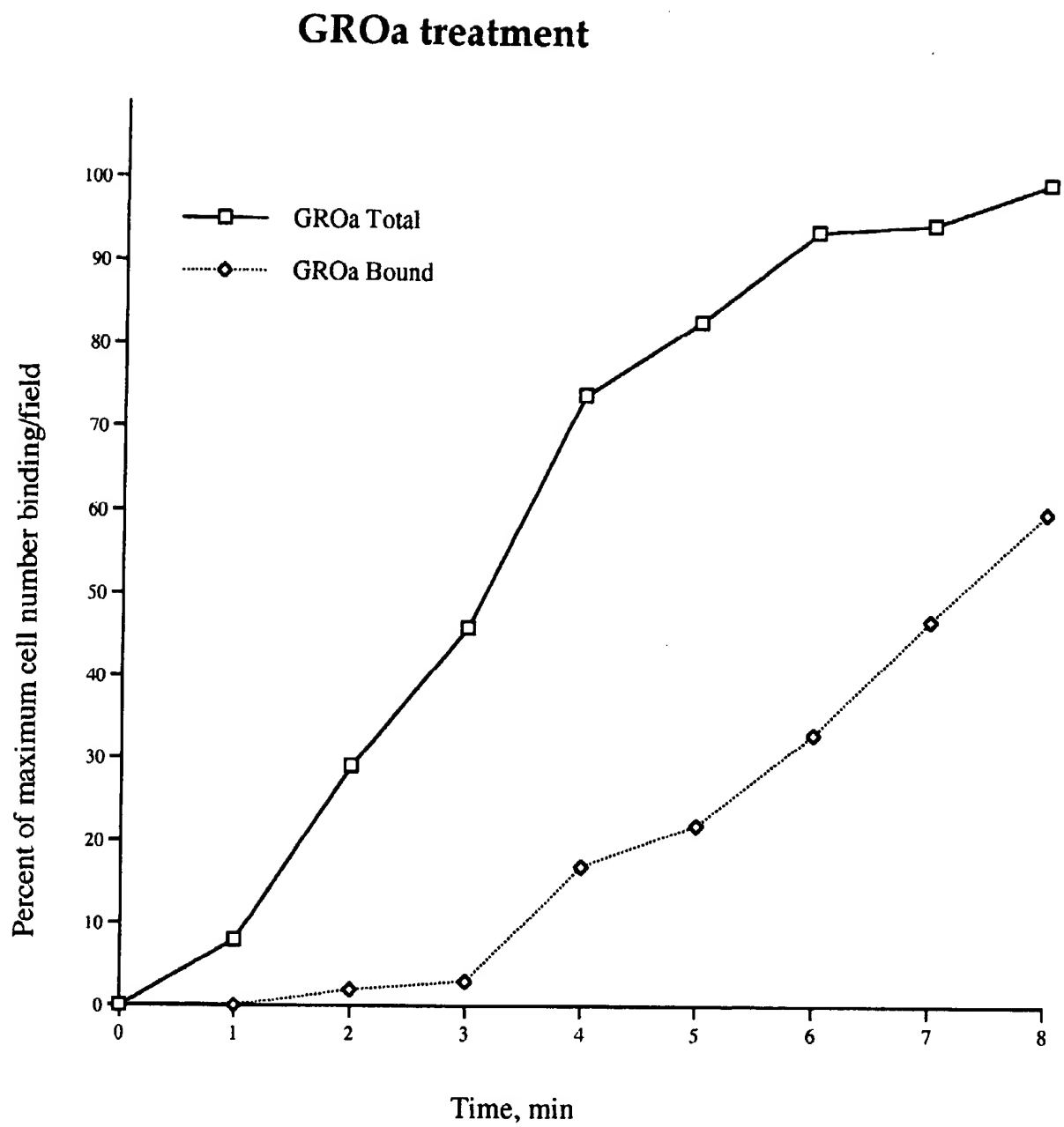


Figure 7

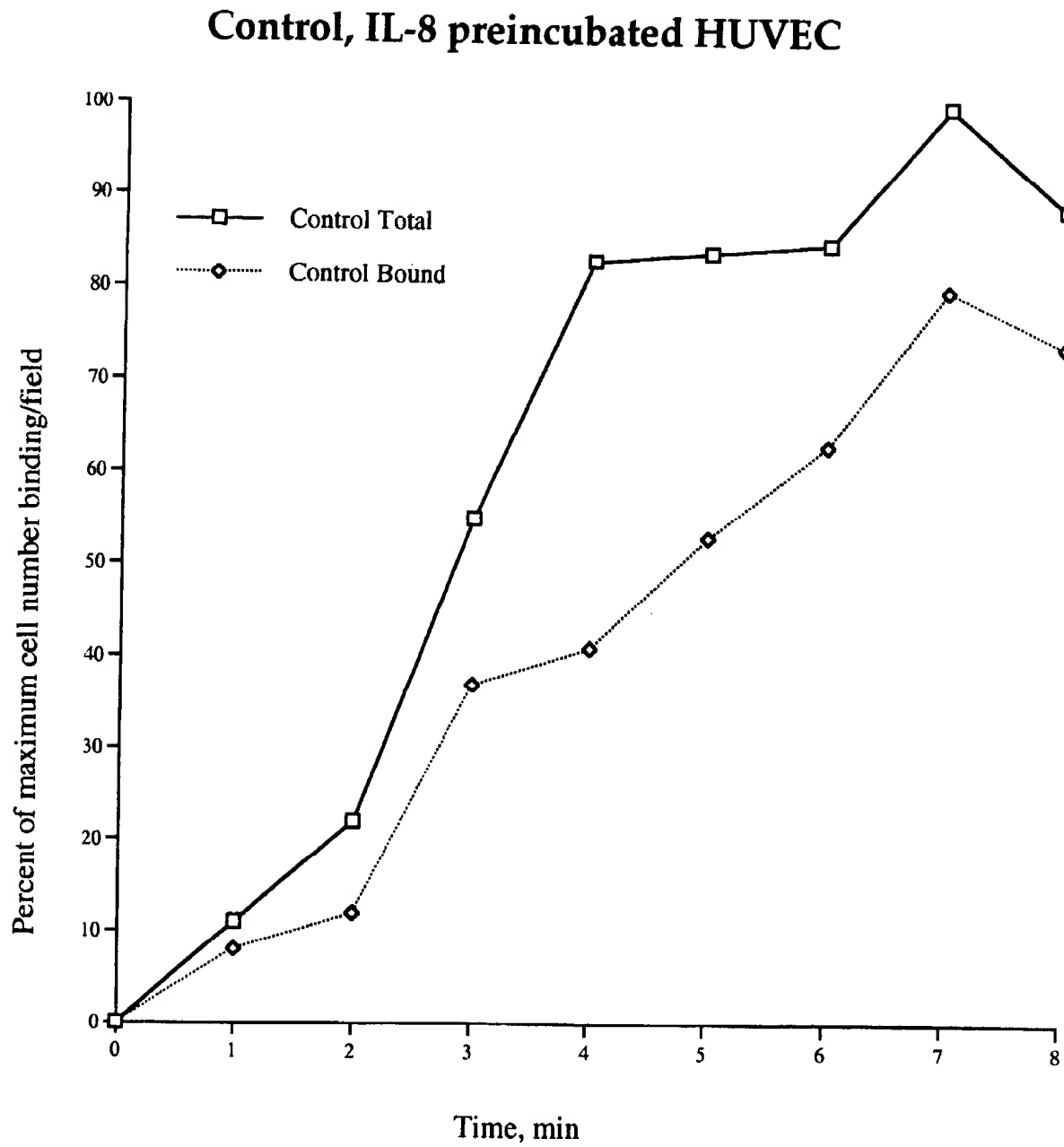


Figure 8

**SB 237844 treated neutrophils on IL-8
pretreated HUVEC**

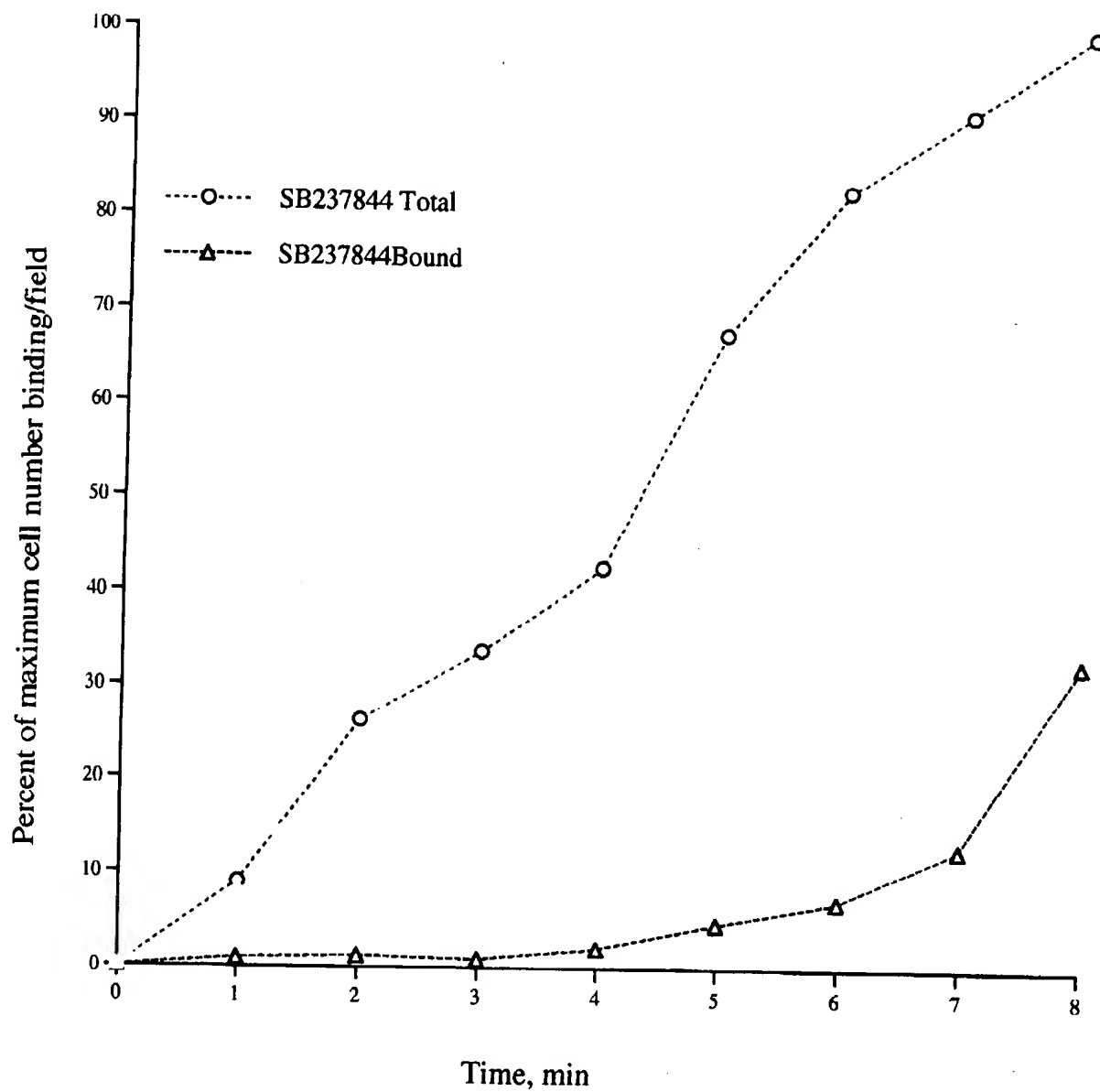


Exhibit B

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T-cell migration; response effected by SB-237844

1. Donor: John White (immunized 7-28-97 w/ tetanus boost)
 A. set up culture 10-3-97; 1.0 ml blood \rightarrow 1.76×10^6 lymphocytes
 B. set up 2 x T-150 flasks w/ 20 ml each + 1:800 FCS

2. After five day culture \rightarrow 86×10^6 cells
 A. Use 5×10^6 cells to stain for CXCR1 vs CXCR2 for FACS
 B. For chemotaxis, need 80×10^6 cells

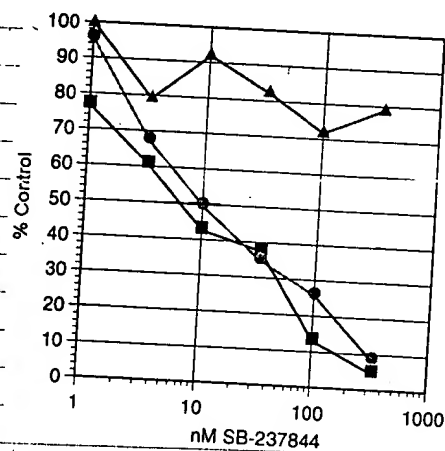
3. Agonists:
 5 nM IL-8, 15 nM GRO- α , 5 nM MCP-1

4. Buffer: RPMI + 0.5% FCS

5. SB-237844 stock in 100% DMSO

| Final | 500 x | 50% DMSO: 50% Buffer | Final |
|--------|--------------|-------------------------------------|--|
| 330 mM | 165 μ M | 6 μ l of 28 mM stock + 0.994 ml | 2.4 ml \rightarrow 0.998 ml buffer \downarrow 0.1% DMSO final in assay |
| 100 mM | 50 μ M | 0.3 ml of 165 μ M + 0.7 ml | |
| 33 mM | 16.5 μ M | 0.1 ml of 165 μ M + 0.9 ml | |
| 10 mM | 5 μ M | 0.1 ml of 50 μ M + 0.9 ml | |
| 3.3 mM | 1.65 μ M | 0.1 ml of 16.5 μ M + 0.9 ml | |
| 1 mM | 0.5 μ M | 0.1 ml of 5 μ M + 0.9 ml | |

Effect of SB-237844 on human T-cell migration in response to 5 nM IL-8, 15 nM GRO- α , or 5 nM MCP-1



IL-8
 GRO- α
 MCP-1

IC₅₀
 6.5 nM
 10 nM
 > 330 nM

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SCIENTIST SIGNATURE:

WITNESS SIGNATURE:

Judy Lee

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DATE:

10-13-97

DATE:

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Effect of SB-237844 on human T-cell migration in response to 5 nM IL-8, 15 nM GRO-a, or 5 nM MCP-1
J. White donor; 5 day tetanus toxoid stimulation

| 10/8/97 | | | | | average | C.I. | % Control |
|--------------|-------|-------|-------|-------|---------|--------|-----------|
| nM SB-237844 | IL-8 | IL-8 | IL-8 | IL-8 | IL-8 | IL-8 | |
| 1 | 261 | 248 | 256 | 255 | 5.80 | 76.73 | |
| 3.3 | 197 | 213 | 222 | 211 | 4.79 | 60.61 | |
| 10 | 178 | 155 | 151 | 161 | 3.67 | 42.67 | |
| 33 | 135 | 138 | 169 | 147 | 3.35 | 37.58 | |
| 100 | 81 | 61 | 98 | 80 | 1.82 | 13.09 | |
| 330 | 49 | 57 | 65 | 57 | 1.30 | 4.73 | |
| 5 nM IL-8 | 277 | 371 | 309 | 319 | 7.25 | 100 | |
| blank | 33 | 46 | 52 | 44 | 0.99 | | |
| nM SB-237844 | GRO-a | GRO-a | GRO-a | GRO-a | GRO-a | GRO-a | % Control |
| 1 | 272 | 276 | 268 | 272 | 6.63 | 95.45 | |
| 3.3 | 240 | 203 | 169 | 204 | 4.98 | 67.36 | |
| 10 | 203 | 146 | 134 | 161 | 3.93 | 49.59 | |
| 33 | 142 | 120 | 114 | 125 | 3.06 | 34.85 | |
| 100 | 87 | 105 | 118 | 103 | 2.52 | 25.76 | |
| 330 | 68 | 52 | 64 | 61 | 1.50 | 8.40 | |
| 15 nM GRO-a | 290 | 275 | 283 | 283 | 6.89 | 100.00 | |
| blank | 48 | 49 | 26 | 41 | 1.00 | | |
| nM SB-237844 | MCP-1 | MCP-1 | MCP-1 | MCP-1 | MCP-1 | MCP-1 | % Control |
| 1 | 377 | 267 | 278 | 307 | 5.49 | 99.74 | |
| 3.3 | 245 | 264 | 250 | 253 | 4.52 | 78.17 | |
| 10 | 318 | 299 | 246 | 288 | 5.14 | 91.93 | |
| 33 | 289 | 254 | 247 | 263 | 4.70 | 82.28 | |
| 100 | 295 | 244 | 172 | 237 | 4.23 | 71.83 | |
| 330 | 256 | 265 | 243 | 255 | 4.55 | 78.84 | |
| 5 nM MCP-1 | 305 | 336 | 284 | 308 | 5.51 | | |
| blank | 63 | 53 | 52 | 56 | 1.00 | | |

* cells counted @ 400 X magnification

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